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BOSTON UNIVERSITY

GRADUATE SCHOOL

Thesis

METHODS OF LIPID ANALYSIS

submitted by

Mildred Elizabeth Huntsinger

(B.S., Simmons, 1923)

In partial fulfilment of requirements for

the degree of Master of Arts.

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Methods of Lipid Analysis.

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This report represents a correlation and criticism of the numerous methods proposed for the quantitative estimation of the various lipids found in the animal organism. The data presented have been compiled from a comprehensive review of the literature and from the results obtained from the author's personal experimental investigations. The term lipid has been agreed upon by the American Society of Biological Chemists (68) to express the fat and fat-like compounds present in the animal body. The lipids, cholesterol, lecithin and fatty acids will be discussed in this report. The author's investigations have been largely limited to devising and using methods applicable to the study of the lipids contained in small amounts of material. For this reason the present communication will be chiefly confined to the discussion of such methods.

Prior to 1908 the character of the methods for lipid analysis was such that the data obtained are of insignificant value for purposes of the present report. In 1908, Kumagawa and Suto (50) made a comprehensive investigation of the subject of lipid analysis and developed much more accurate methods. In 1909, Windaus (87) developed a procedure for the determination of cholesterol and cholesterol esters. Later, in 1912, Bloor published methods for lipid analysis which were applicable to the study of the living animal. All these investigations stimulated greater interest both in the study of lipid metabolism and the further development of methods for such studies.

The results of such investigations can be obtained only by correlating reports published in numerous chemical, biochemical and clinical journals. The published books on lipids deal only indirectly with the subject of this thesis (52, 59).

When animal and vegetable tissues are extracted with such organic solvents as alcohol or ether, the soluble material consists of ordinary fat together with a variable amount of other substances. Many of these latter substances are of fatty nature and yield fatty acids on hydrolysis, but differ from neutral fat in that they contain nitrogen, or nitrogen and phosphorus in the molecule. Others of them bear no resemblance whatsoever to fat except the accidental similarity of solubility in the ordinary fat solvents. It is difficult to isolate individual lipids in chemically pure form since they usually occur in mixtures and many are intersoluble.

The terms used to define material extracted as described are mostly indefinite as to just what chemical substances are included. Leathes and Raper (52) define the term lipin as denoting substances of a fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids, and containing in their molecule either nitrogen, or nitrogen and phosphorus. Phospholipins is used to denote compounds of fatty acids that contain phosphorus and nitrogen. Compounds of fatty acids that contain nitrogen and galactose are referred to as galactolipins. Sterols are not fats, but substances of a fat-like nature. They have been

included in the lipid group only because they are soluble in the ordinary fat solvents, and because they form esters with fatty acids.

Bloor (14) used the term lipoids as a general term for all those substances connected with the metabolism of the fatty acids, including the fatty acids, their naturally occurring compounds, and such substances as cholesterol which occur naturally in combination with the fatty acids and which are therefore presumably connected with their metabolism. In 1921, Bloor classified lipoids as follows:

1. Simple lipoids
   a) Fats
   b) Waxes
2. Compound lipoids
   a) Phospholipoids
   b) Glycolipoids
3. Derived lipoids
   a) Fatty acids
   b) Sterols

In later publications Bloor uses the term lipid instead of lipoid (25).

MacLean (59) classifies the fatty substances found in the animal organism as follows:

A. Neutral fat and fatty acids

B. Phospholipins or Phosphatides: substances containing fatty acids, nitrogen and phosphorus. (i.e. lecithin)

C. Galactolipins or cerebrosides: substances

containing fatty acids, nitrogen and a carbohydrate group but no phosphorus.

D. Substances of varying chemical nature having no relation to fats (i.e. cholesterol).

Bang has classified lipoids as:

I. Those containing carbon, hydrogen and oxygen,

a) lipoids of the aliphatic series

(1) fats

(2) fatty acids

b) lipoids of the aromatic series

(1) sterols, as cholesterol

II. Those containing carbon, hydrogen, oxygen and nitrogen,

a) phosphatides, --- lecithin, cephalin

b) cerebrosides, --- phrenosin, kerasin.

For purposes of description in the present thesis, the term lipid will be used to include all the fatty substances in the animal organism. Fatty acids, lecithin and cholesterol are the lipids which will be discussed.

Fatty Acids.

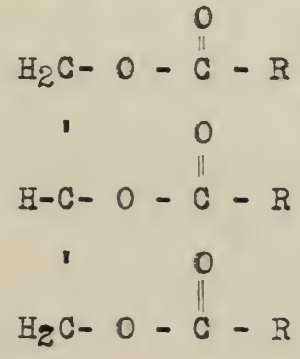
Fat is present in all the tissues of the body. In the active organs such as the liver, heart and kidney, the fat is mainly composed of unsaturated glycerides. The more saturated fatty acids are in the adipose tissue and consist almost entirely of triglycerides made up of fat

synthesized by the body from carbohydrate (52), and from the excess fat from food. It is likely to vary in composition being primarily influenced by the character of the fat in the food. The fat content of the adipose tissue of any one species of animal is approximately constant in composition and has an iodine value that does not vary greatly from the characteristic average value. The lower melting fats have a higher iodine value, and therefore contain a greater proportion of unsaturated fatty acids. There appear to be grounds for attributing the well marked differences in composition of adipose tissue fat from different parts of the same animal, to the fact that it is deposited in regions of different temperature.

The synthesis of fat from carbohydrate presumably takes place in two stages: (1) the building up of the fatty acid, and (2) the union of three molecules of the fatty acid with glycerol to form a triglyceride. It is thought that this union of fatty acids with glycerine to form fat may be brought about by the agency of the enzyme lipase, which according to the conditions in which it acts, can either catalyse the hydrolysis of fat or its synthesis. Possible explanations are suggested by Leathes and Raper (52). Fats are formed in nature by a series of reactions the end points of which vary according to the temperature at which they occur. The fatty acid chains contain an even number of carbon atoms, hence must be built up two at a time. Some are unsaturated in varying degree, while

some are fully saturated. The process must be one which may yield either unsaturated or saturated fatty acids with slight changes of condition. Since saturated fatty acids contain a greater store of potential energy than unsaturated fatty acids, it is probable that fats are stored in the former condition in the animal organism, and are oxidized as needed to unsaturated fatty acids. This "desaturation" of fatty acids is thought to take place in the liver. There is a strong presumption on chemical grounds that the glycerol, which combines with the fatty acid in the synthesis of fat, is formed from carbohydrate. Although many points remain to be cleared up regarding fat synthesis in the animal organism, it is known that fat is synthesized by the body and stored in the adipose tissue as a source of energy.

The molecular structure of a fat is expressed by the structural formula:



in which R is the fatty acid radical. R may be the same or different. The basis of the fat is glycerol. Since R may vary, there are a great many possible combinations. Hence there are a great many fats of varying physical and chemical properties.

The fatty acids are divided into two major classes: (1) saturated, and (2) unsaturated. A saturated fatty acid is an aliphatic monocarboxylic acid with no double or triple bonds. An unsaturated fatty acid is an aliphatic monocarboxylic acid which contains one or more double or even triple bonds. A table of the saturated fatty acids is given by Leathes and Raper (52). It is included here for completeness.

Table of the Saturated Fatty Acids. (52)

	Formula	Mol. Wt.	Melting point	Boiling point	Specific gravity
Formic	$HCOOH$	46	+ 8.6	100.8	1.2415 at 0°
Acetic	CH_3COOH	60	16.5	118	1.0701 "
Propionic	C_2H_5COOH	74	- 22	140.9	1.0133 "
n.Butyric	C_3H_7COOH	88	- 6.5	163.5	0.9781 "
Isobutyric	"	88	- 47	155.5	0.9651 "
Caproic	$C_5H_{11}COOH$	116	- 20	204.5	0.945 "
Caprylic	$C_7H_{15}COOH$	144	16	236	0.927 "
Capric	$C_9H_{19}COOH$	172	31.3	270	0.930 at 37
Lauric	$C_{11}H_{23}COOH$	200	48.0		0.875 " 43.5
Myristic	$C_{13}H_{27}COOH$	228	58.0		0.862 " 53.6
Palmitic	$C_{15}H_{31}COOH$	256	63.5	340 to 356	0.853 " 62.6
Stearic	$C_{17}H_{35}COOH$	284	71	360	0.845 " 69.3
Arachidic	$C_{19}H_{39}COOH$	312	76.7		
Lignoceric	$C_{23}H_{47}COOH$	368			
Cerotic	$C_{25}H_{51}COOH$	396	78		
Melissic	$C_{29}H_{59}COOH$	452	91		

The saturated fatty acids have the general formula $C_nH_{2n}O_2$. The naturally occurring members usually contain an even number of carbon atoms. Those containing less than ten carbon atoms are fluid at ordinary temperature, while those containing more are solid. The most common of the saturated fatty acids are palmitic, $C_{16}H_{32}O_2$, and stearic, $C_{18}H_{36}O_2$.

The unsaturated fatty acids may be of:

1. The oleic acid series, having the general formula $C_nH_{2n-2}O_2$, of which oleic acid is the best known member. The formula for oleic acid is $C_{18}H_{34}O_2$. It has one double bond.
2. The linoleic acid series, having the general formula $C_nH_{2n-4}O_2$. The fatty acids in this series have two double bonds.
3. The linolenic acid series, having the general formula $C_nH_{2n-6}O_2$. The fatty acids in this series have three double bonds.
4. There probably are other fatty acids having four or more double bonds which have not been certainly identified up to the present. A very few fatty acids having four double bonds are mentioned in the literature.

The lower melting fats have the higher iodine numbers and therefore contain a greater proportion of unsaturated fatty acids. The more unsaturated a fatty acid or its glyceride is, the greater its unstability. The more unsaturated fatty acids are burned first in the body.

Oleic acid occurs as a glyceride in most fats and oils, and generally occurs in larger quantities than any other single fatty acid. It is insoluble in water, somewhat soluble in cold, and freely soluble in hot alcohol. It is very soluble in ether. Oleic acid absorbs two atoms of halogens due to the double bond in the middle of the chain. Hartley (40) found that the oleic acid in the liver is different from the oleic acid found in olive oil. In the oleic acid from the liver, the double linkage lies between the 6th and 7th carbon atoms, reckoned from the methyl group end of the chain. Of the linoleic acid series, the acid from linseed oil is the one most studied, but even its constitution remains to be determined. The linolenic acid series contains the higher unsaturated fatty acids. Here, also, much remains for future investigation.

The unsaturated fatty acids may be converted into saturated fatty acids by hydrogenation. They react readily with halogens under suitable conditions and add halogen atoms in proportion to the degree of unsaturation. This degree of unsaturation is obtained by determination of the iodine number, which is the number of grams of iodine that is absorbed by one hundred grams of the fat. The iodine number is therefore an index of the degree of unsaturation. For example, oleic acid, which contains one double bond, can absorb two atoms of iodine or bromine. The theoretical iodine number of oleic acid on this basis is 90.1

Fats are hydrolyzed by the action of acids, alkalies, fat splitting enzymes (lipases), and superheated steam into three molecules of fatty acids and one molecule of glycerol. When treated with alkali, fats form glycerol and the salts of fatty acids (soaps). This is termed saponification. The saponification values of the various fats have been determined and are of value in serving as a measure of the mean molecular weight of the fatty acids that are present in the fat, those of lower molecular weight neutralizing a larger weight of the alkali. The saponification number of a fat is the number of milligrams of potassium hydroxide neutralized by the saponification of one gram of fat. In a broader sense, saponification is the hydrolysis of any ester with or without alkali.

Biological Material Used.

The biological materials used for lipid studies are foodstuffs, blood, duodenal contents, tissues and feces. The lipid content of blood may be determined in whole blood, plasma or serum, and indirectly in the corpuscles by calculation. The usual procedure is to extract the lipid material with an organic solvent such as alcohol or ether or an alcohol-ether mixture, evaporate the solvent and weigh the lipid residue. According to the type of material used, the steps in its preparation prior to extraction vary.

Preparation of Material.

In the determination of lipids in whole blood, an

alcohol-ether extract of the blood is obtained as follows. Five cc. of blood are run slowly and with shaking into approximately 60 cc. of the alcohol-ether mixture (75 cc. of redistilled alcohol and 25 cc. of redistilled ether), in a 100 cc. volumetric flask. Then complete extraction is accomplished by heating to boiling by immersing in a hot water bath. After cooling, it is made up to volume with alcohol-ether, mixed and filtered. The determinations are run on this extract (22).

The only lipid determined in duodenal contents (63) is cholesterol. This is a useful routine test in the determination of the functional state of the liver. 10 cc. of duodenal contents are added to 50 cc. of a mixture of alcohol, ether and chloroform. The foreign organic matter is removed by the addition of potassium permanganate, the excess of which subsequently is removed by potassium metabisulphite. Any water present is separated by the addition of anhydrous sodium sulphate. After filtering, a sample of the filtrate is taken and the estimation of cholesterol is carried out as described by McClure and Huntsinger (63).

In tissues, the lipid content is obtained by estimation of the amount of fatty acids which they are capable of yielding after saponification. The older methods reported as fat the ether soluble fraction. These results were not accurate since the values obtained in this way included substances other than fat. The later methods depend upon extraction and saponification,

by which the error due to cholesterol is eliminated although that due to cholesterol esters remains. Thus the marginal error is substantially reduced. The animal tissue is first minced, and then dried before it can be extracted. The drying is accomplished by means of (1) air or some neutral gas, (2) alcohol, (3) salts, such as anhydrous sodium sulphate or calcium sulphate. The dried material must then be powdered, after which it can be extracted with ether.

Studies on the fatty compounds present in stools are reported in relation to investigations of both normal and abnormal metabolic states. For this purpose the collected stools are dried on the water bath, pulverized and extracted with one of the usual fat solvents. An apparatus most commonly used for this extraction is the reflux condenser devised by Soxhlet or one of the many later modifications. Methods for the determination of lipid in feces are given by Folin and Wentworth (36) and by Sperry (82).

Quantitation of the Lipids.

The chemical methods used for quantitating lipids are volumetric, gravimetric, colorimetric and nephelometric. Gravimetric methods necessitate isolation of the required substance in pure form, which is usually tedious and requires much time and material. Therefore,they are often not adaptable to the requirements peculiar to the study of living animals. On the other hand, many colorimetric and some nephelometric and volumetric methods have been developed which more nearly meet the requirements of practice.

Colorimetric methods have come into general use because of their relative simplicity and accuracy if certain limiting conditions be observed. The nephelometer offers equal simplicity of operation but is subject to sources of error intrinsic in the method itself. The difficulty of procuring the physical conditions necessary for the formation of a uniform suspension of the material under investigation is obvious. Furthermore, the eye is more sensitive to minute changes in color than to intensity of light. Finally, while the color intensities are proportional to concentrations only within a limited range, these boundaries are far more liberal than those regulating shadow effects. The fatty acids may be determined nephelometrically according to Bloor (22). The colorimeter may be used in the estimation of cholesterol directly and of lecithin indirectly. Volumetric methods are used in obtaining the iodine numbers of both cholesterol and fatty acids.

Nephelometric and colorimetric methods require the use of solutions of chemicals of known concentrations, which are technically called standards. The comparison of these standards with similar solutions of unknown concentration gives a means for determining the quantity of material in the latter. Such comparisons are made by physical instruments bearing the name of the process.

Methods for the Determination of Fatty Acids.

Since fatty acids are essential metabolic constituents of the blood lipids, their determination along with that of cholesterol gives a measure of its gross lipid content. When time and material are available these substances are best determined gravimetrically, by which method they are isolated and weighed. The method of Kumagawa and Suto (50) is the standard macromethod. In this the tissue is destroyed and the fats saponified with potassium hydroxide, the fatty acids are set free by adding an excess of hydrochloric acid and they are then extracted with ether.

For most purposes, the gravimetric methods are too tedious and the various micromethods are preferred. In 1922, Bloor (22) published a nephelometric method for the determination of fatty acids and cholesterol in small amounts of blood plasma. This depends upon the extraction of lipid material from the blood by alcohol-ether, saponification of the extracted lipid with alkali, and determination of the cholesterol and fatty acid content. In the procedure the cholesterol is separated from the fatty acids by the fact that dry soaps are practically insoluble in cold chloroform while cholesterol dissolves readily.

For the determination ten cc. portions of the alcohol-ether extract of blood, prepared as described previously, are measured into small beakers of 100 cc. capacity. 0.1 cc. of concentrated sodium hydroxide, made from sodium, is added and the mixture evaporated on the water bath until only two or three drops remain. The alkali is then par-

tially neutralized by the addition of 0.1 cc. of dilute sulphuric acid (1 of acid to 3 of water), and the liquid well mixed and distributed over the bottom of the beaker. It is then evaporated just to dryness. After cooling, the cholesterol is extracted in the cold with successive portions of chloroform, the combined extracts being used for its quantitative determination to be described later. To the residue in the beakers after the extraction with chloroform, is added 10 cc. of redistilled alcohol. The mixture is allowed to boil on the electric stove for ten minutes. The hot alcohol is the poured through the same small filter paper, that was used in the extraction of the cholesterol, into a beaker of 100 cc. capacity. The extraction is repeated once, pouring the hot extracting fluid through the filter into the beaker. The combined extracts are evaporated to a volume of 2 to 3 cc., then transferred quantitatively to a small graduated, glass-stoppered cylinder, the beaker rinsed out with alcohol, and the washings used to bring the volume up to 5 cc. One hundred cc. of distilled water are measured into a 200 cc. beaker and the alcoholic extract of fatty acids is added, with stirring, through a small funnel with the stem drawn out to form an opening of about 1 mm. in diameter. The cylinder is rinsed once with the solution in the beaker, and the rinsings are poured back into the beaker through the funnel. To another beaker containing 100 cc. of distilled water are added through a pipette, with stirring, 5 cc. of the alcoholic standard containing

in all 2 mgm. of a mixture of 60 % oleic and 40 % palmitic acids. Ten cc. of dilute hydrochloric acid (1 of acid to 3 of water) are added to each beaker, with stirring and after standing for five minutes the unknown and control solutions are compared in the nephelometer. This method requires a knowledge of the nephelometer and a large amount of practice and experience in its use. Further, unless the two concentrations are nearly the same, serious errors are inevitable.

Bloor's nephelometric method has been criticized by Blix (9) on the grounds that (1) it utilizes an instrument, the nephelometer, which is not available in every laboratory and which requires some experience in manipulation, (2) the conditions on which the method depends do not allow flexibility when large amounts of fat are absorbed from the intestine, and (3) the results are approximate rather than absolute.

The nephelometric method of Bloor depends upon the maintenance of a proper acidity. If the fatty acids determined are oleic and palmitic acids the method is more accurate than when other fatty acids are present. For use in a clinical way, the method does show increases in amounts of fat present in the blood. Where exact amounts are desired the oxidation methods are better, since they are more flexible and more accurate.

In 1918, Bang published an oxidation method for the determination of fatty acids. In this method the fat is oxidized with dichromate and sulphuric acid, and the estimations are made from the amount of dichromate reduced.

In 1926, Blix (9) published a micromethod for the determination of blood lipids which is in reality a modification of Bang's procedure. The modified method as outlined is very tedious, and though the results are more accurate, the method is too detailed and too exacting for practical purposes.

Bang's method has not only been modified by Blix but also by other workers in this field. Each modification involves a different set of factors in the final calculation of the oxidation values. Therefore, the results of any single method will give consistent relative values, but those of two different methods are usually not mutually comparable. Only a standard method of procedure based on complete oxidation could be relied on to give absolute values. Bloor investigated Bang's method and was unable to obtain satisfactory results. This he found was due to incomplete oxidation of fatty acids. For this reason Bloor modified the method using a larger amount, the basic weight chosen being 2 mgm. The general plan was to heat the fatty substance with an excess of sulphuric acid-dichromate oxidizing agent and to determine the amount of reagent used and so the amount of material oxidized. This was done by titrating the unused reagent with standard sodium thiosulphate solution. By adding a catalyst (silver dichromate) to the reagent, Bloor found that it was possible to shorten the time of heating and also to increase the certainty of the determination. He found that both the time and temperature factors were extremely important in securing complete oxidation of the fatty

substance. Using pure substances, Bloor determined the proper conditions, such as temperature and time factors, prior to applying the method to the alcohol-ether extract of blood. The preliminary results obtained with pure substances, were found to be within a few percent of the absolute values, when the modified procedure is followed.

The results obtained by the oxidation method of Bloor (25), are highly accurate. The procedure is as follows. The total fatty acids and cholesterol are obtained by extraction with alcohol-ether and are saponified by boiling with sodium ethylate. The whole is then evaporated to dryness, the residue acidified and extracted with petroleum ether. An aliquot portion of the petroleum ether extract is evaporated to dryness and the oxidation value of the sodium soaps and cholesterol is determined. This is accomplished by the addition of a silver dichromate solution in sulphuric acid, adding an excess of potassium iodide solution when the oxidation is complete and titrating the iodine liberated by the remaining dichromate with thiosulphate solution, using starch solution as an indicator. Accuracy with the above method can only be obtained by the strictest adherence to the technic outlined by Bloor. He states that the method is exactingly quantitative and recommends that a proper technique be developed by using it first on known amounts of known substances, before attempting to apply it to the estimation of unknown materials. The method obviates all objections to the earlier modifications of the Bang technic. The results

by it are comparable to the nephelometric values, except that the oxidation method gives somewhat higher values. In the discussion Bloor, who has been an active contributor in this branch of biochemistry states that from an examination of the literature and from his personal experience the accurate determination of the fatty substances of the tissues and fluids is never an easy procedure and that this is particularly the case with micromethods.

Two other methods may be considered here, one a colorimetric procedure, and the other a microtitration. In 1925, Stewart and White (84) published a method for the estimation of the fat in the blood by microtitration using pure palmitic acid as a control, and then applied it to the alcohol-ether extract of blood. The microburette of Rehberg (73), which delivers 0.10 cc. with extreme accuracy, is used to titrate the fatty acids liberated by hydrolysis of the fat contained in 1 cc. of blood. This method is hardly adapted to routine measurements and is primarily for research use. The procedure given is as follows; 25 cc. of alcohol-ether extract of blood are taken, the ether is evaporated off, and 5 cc. of N/10 sodium hydroxide and 5 cc. of absolute alcohol are added. The mixture is evaporated almost to dryness on the water bath, (this takes two hours), Then after hydrolysis, 5 cc. of N/10 hydrochloric acid are added to the mixture. This theoretically neutralizes the whole of the sodium hydroxide and liberates quantitatively the fatty acids produced during the hydrolysis. The solution is then

boiled down to about 1 cc. in order to drive off absorbed carbon dioxide. The residual liquid is transferred quantitatively to a 10 cc. volumetric flask, absolute alcohol being used for the washings and for making up to a volume of 10 cc. One cc. of this final solution is titrated with N/10 sodium hydroxide using the Rehberg burette. The sodium hydroxide used must be free from carbonate. All reagents and apparatus must be very carefully standardized.

A colorimetric micromethod for the determination of fats and lipins in blood was published by Milroy in 1928. The method is based on the principle of saponification of the compounds in the blood containing fatty acids and the subsequent determination of the amount of fatty acids isolated from the products of saponification. The colorimetric estimation depends on the formation of a colored salt of the isolated fatty acids of the blood with the base of Nile-blue hydrochloride.

These methods for the estimation of fatty acids which have been discussed are those principally used for the determination of fatty acids in small amounts of blood. When time and material are available fatty acids are best determined gravimetrically. However, for most purposes, the gravimetric methods are too tedious and one of the other methods is more practical. Where exact amounts are desired the oxidation methods are better since they are more flexible. For use in a clinical way, Bloor's nephelometric procedure may be used since the results obtained,

even although they are relative rather than absolute, do show increases in amounts of fat present in the blood. Bloor's oxidation method should be used where extreme accuracy is desired.

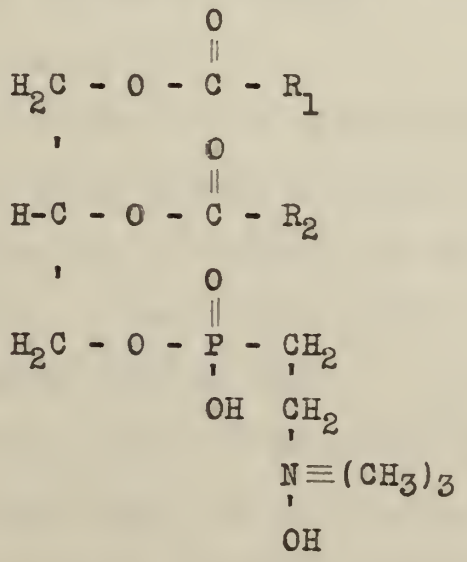
McClure and Huntsinger (64) found Bloor's nephelometric method satisfactory for the estimation of total fatty acids in blood, known amounts of fatty acids being quantitatively recovered. Normal values for total fatty acids in the blood are reported by Bloor as from 290 to 410 mgm. per 100 cc. of blood for normal men. McClure and Huntsinger (64) found normal values to range between 283 to 442 mgm., the average value being 364 mgm. In pathological cases the values may vary from the normal level, and such values have been reported in hyperlipemia, diabetes, nephritis and in obstructive jaundice. In these conditions marked increases above the normal values for total fatty acids have been found.

Phosphatides.

Phosphatides are compounds of fatty acids that contain phosphorus and nitrogen. They occur with the fats in nature; appear to be present in every animal and vegetable cell. They have distinct fat-like properties and are especially abundant in the brain, heart, muscles and liver. The nature of these fatty acid complexes, rich in phosphorus, is very uncertain and awaits further investigation. Some of the properties of the phosphatides are known through important extensive investigations carried out among others by Levene (55). However, much remains to be learned from the study of decomposition products of lecithin and cephalin. This study should through light on the metabolic history of the phosphatides and also on the fatty acids.

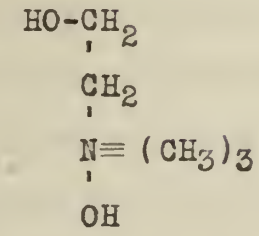
Conventional formulas are assigned as follows:

(1) lecithin has the structural formula:

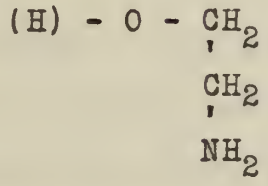


in which R_1 and R_2 may be either unsaturated or saturated fatty acids. In the above, choline is con-

jugated to the phosphoric acid radical. Choline has the formula:



(2) Cephalin has in place of the choline radical, amino ethyl alcohol.



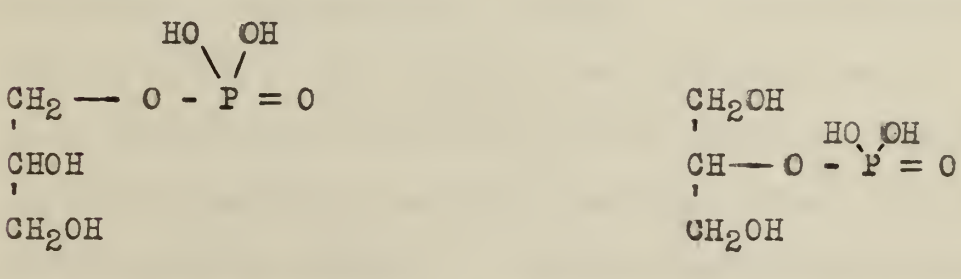
Phosphatides are soluble in the ordinary solvents for fats, but are practically insoluble in acetone. They are classified according to their nitrogen-phosphorus ratio. In lecithin and cephalin this ratio is 1 : 1 Both contain fatty acids of the unsaturated series, hence are liable to oxidation changes. The nitrogen-phosphorus ratio of sphingomyelin is 2 : 1 It contains saturated fatty acids, hence it is a stable substance.

Lecithin when freshly prepared is a yellowish-white waxy substance but on exposure to air it soon becomes dark brown in color. The melting point is indefinite (60° ±); it decomposes at 110° C. (59) It is very hygroscopic. It is readily soluble in alcohol, ether, chloroform, benzene, petroleum ether, as well as in aqueous aolutions of "bile salts", but is insoluble in acetone. In contact with water it forms a colloidal solution. Lecithin owes

many of its chief properties to the ease with which it enters the colloidal state.

Lecithin is composed of choline, glycerophosphoric acid and two fatty acids, one saturated and one unsaturated. It absorbs iodine and bromine, but constant iodine or bromine values can not be expected since lecithins from different sources contain different unsaturated fatty acids having one or more double bonds. On hydrolysis it breaks down into glycerophosphoric acid , fatty acids and choline. The glycerophosphoric acid is partly decomposed to glycerol and phosphoric acid. Glycerophosphoric acid can exist in two modifications, the α and β forms.

α form (unsymmetrical) β form (symmetrical)



The α form contains an asymmetric carbon atom and is optically active. Levene (55) working on pure lecithin established the fact that the laevorotatory form of glycerophosphoric acid does occur. Whether or not any of the dextrorotatory form actually occurs in pure lecithin is unknown. Both lecithin and cephalin contain the laevorotatory form of glycerophosphoric acid.

In 1915 MacLean (59) found that lecithin as then prepared contained cephalin as a contaminant. Experiments had suggested that lecithin was really a mixture of two substances of somewhat similar composition. Further investigation led to the conclusion that cephalin was present with pure lecithin in varying proportions in what was then termed "lecithin". Observations on the iodine numbers suggest the possibility that at least one of the fatty acid radicals in the lecithin molecule is unsaturated. Observers agree that both saturated and unsaturated fatty acids may occur in the same molecule. Levene (55) has shown that they occur in equal amounts. Attempts to synthesize lecithin have so far given only unsatisfactory results.

The usual procedure for quantitating lecithin is to determine its phosphorus content. Several methods have been devised. The earlier ones were gravimetric. However, these necessitated the use of relatively large amounts of material and this tended to limit their use for biological studies. In 1914 Taylor and Miller (85) devised a colorimetric method. Their procedure was modified by various investigators, notably Bell and Doisy (3), later by Hess and Helman (42), then by Fiske (35) and more recently by Leiboff (53). These modifications have finally yielded greatly improved methods for the colorimetric estimation of phosphorus, which are applicable to the estimation of small amounts.

To isolate and weigh the phosphatides is hard. The

majority of results obtained in attempts to estimate the phosphatide content of tissues have been reached by extraction of the tissue with suitable organic solvents and estimation of the phosphorus content of the more or less purified extract. Extraction with ether alone is unsuitable, since phosphatides are seemingly present in some kind of combination with the cell proteins, and only a small amount of the phosphatide material is dissolved. Practically all methods, both old and new, depend on extraction with alcohol and ether. In the methods for obtaining the phosphatides, the tissue is first freed from macroscopic fat and then dried. This is not easy as the drying must be done in the shortest possible time, with the minimum exposure to air and at the lowest temperature that can be conveniently employed. Only in this way can one isolate the phosphatides in an unchanged condition.

The micromethods employ smaller amounts of material and are therefore suitable for the estimation of the lipoidal phosphorus of blood. For the colorimetric procedures the alcohol-ether extract of blood prepared as outlined by Bloor (v.s.) is used. The method of Taylor and Miller (85) depends upon precipitation as ammonium phosphomolybdate and the colorimetric estimation of the precipitated molybdenum by reduction with phenylhydrazine. The procedure of Bell and Doisy (3) depends upon reduction with hydroquinone in the presence of molybdic acid.

The method of Hess and Helman is based upon that of Taylor and Miller, and of Bell and Doisy. The procedure

(42) consists in the extraction of the phosphatides with alcohol-ether mixture and determination of the amount of phosphorus present. The method is rather lengthy and the results do not always check closely, as it was found in an attempt to use the procedure in a study on fat metabolism (64). The method calls for extraction of the blood with alcohol-ether, digestion of the extract with acid; then after neutralization with concentrated ammonia, it is made just acid, and the phosphorus is precipitated with a strychnine solution. One half hour is allowed to insure complete precipitation of the phosphorus. It is then centrifuged, the liquid decanted, the precipitate washed three times with distilled water, and finally dissolved in 2 cc. of a 1 % solution of sodium hydroxide. Distilled water is added to 10 cc., the material is quantitatively transferred to a 100 cc. volumetric flask, 20 cc. of a 20 % solution of potassium ferrocyanide are added and then 10 cc. of concentrated hydrochloric acid are added, measured accurately with a burette. The contents of the flask are mixed, allowed to stand ten minutes , diluted to the 100 cc. mark and read in the colorimeter. The standard, 2.5 cc. of which contain 1 mgm. of phosphorus, is pipetted into a tube and precipitated at the same time as the unknown.

In Fiske's method (35) for the determination of inorganic phosphorus, the blood is precipitated with trichloracetic acid and the filtrate used. Trichloracetic acid precipitates the protein and dissolves the

inorganic and acid soluble phosphorus compounds. For organic phosphorus it was found that Fiske's method could be used on the alcohol-ether extract of blood. This is the only modification necessary to adapt Fiske's procedure for use in the estimation of the lipoidal phosphorus (64). In this method reduction is effected by the use of aminonaphtholsulfonic acid. The procedure is as follows: 10 cc. of the alcohol-ether extract of blood are pipetted into a pyrex tube graduated at 50 cc. The tube is placed in a beaker of boiling water and the contents evaporated just to dryness. Five cc. of 5 N sulphuric acid are added and it is then heated, using a microburner. As soon as charring can be seen the flame is turned down very low so that the mixture barely boils. It is then heated until there is no further blackening. One drop of concentrated nitric acid is added, allowing it to run down the side of the tube. If the color does not promptly disappear another drop of nitric acid is added in the same manner. It is necessary to heat for about 30 seconds more to complete the oxidizing process. The tube is cooled, water is added (about 35 cc.), then 5 cc. of molybdate solution and 2 cc. of the reducing agent are added. After diluting to the 50 cc. mark, it is allowed to stand for 5 minutes before comparing with the standard in the colorimeter. The standard solution used is prepared from commercial monopotassium phosphate. 0.3509 grams are dissolved in a liter of distilled water, so that 5 cc. of the solution equal 0.4 mgm. phosphorus. The standard used for comparison with the test is made as

follows: 2.5 cc. of the standard phosphate solution (containing therefore 2 mgm.) are pipetted into a 100 cc. volumetric flask, 35 cc. of water, 5 cc. of 5 N sulphuric acid, 5 cc. of molybdate solution and 2 cc. of the reducing solution are added. It is then made up to volume. This method was found to be superior technically and to give results more closely approximating the theoretical yield than did other methods.

Bloor (11) determines the lecithin content of blood on aliquot portions of the alcohol-ether extract. In brief the method depends upon digestion with sulphuric-nitric acid mixture, and the subsequent estimation, nephelometrically, as a strychnine phosphomolybdate suspension. This method is open to the usual criticisms for nephelometric procedures.

A colorimetric method for the determination of the lipoidal phosphorus in blood has just been published by Leiboff (54). The procedure uses the alcohol-ether extract of blood. This method is so very recent that it still remains to be tried out by other workers,

Values obtained by McClure and Huntsinger (64) on normal men, using the method of Hess and Helman ranged from 14.0 to 18.3 mgm. lecithin-phosphorus per 100 cc. of blood, while those obtained using Fiske's procedure were between 10.7 and 14.1 mgm. This comparison of the two methods showed that the results obtained by the method of Hess and Helman were often much higher and more variable in the same series of blood specimens. It was found that

the lecithin-phosphorus values were not very far from the normal in patients having disturbances in liver function, ranging from 8.7 to 18.8 mgm. The higher values were obtained in cases of obstructive jaundice. One patient having obstructive jaundice showed a lecithin-phosphorus value of 39.3 to 47.1 mgm. These results were checked by running analyses of other specimens of blood. The cholesterol and total fatty acid contents were also markedly higher than normal.

				Normal
Cholesterol.	392	-	430	140 - 180
Fatty acids	609	-	870	280 - 440
Lecithin-phosphorus	39.3	-	47.1	10.7- 14.0

Cholesterol.

Physiologically, cholesterol is regarded as the most important of the sterols. It was first isolated from gall stones in 1775 by Conradi. Cholesterol derives its name from the fact that it is a constituent of bile. It occurs in practically all animal tissues, being very abundant in brain and nerve tissue, where it exists both free and in combination with fatty acids as esters. It is also found in blood, bile, the skin and other tissues, and is a common constituent of gall stones. It is considered an essential constituent of the living cell.

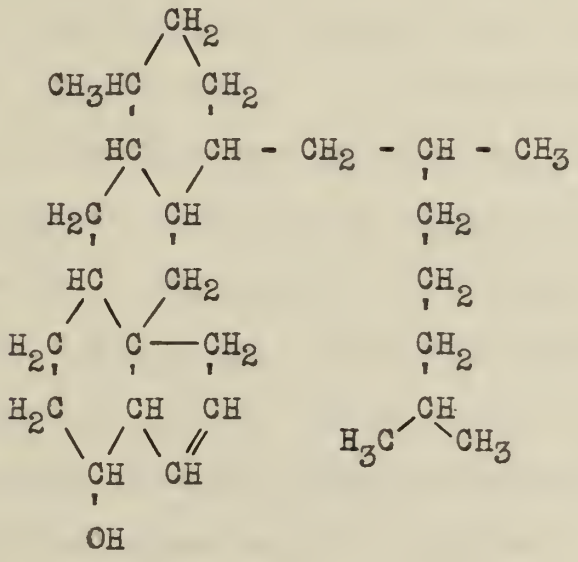
Cholesterol in the animal organism originates mainly from ingested food, the material being present as such or in isomeric forms which can be changed to cholesterol in the body. It is absorbed from the intestines and during

absorption is partly esterified with the fatty acids. Bloor considers it to take an active part in fat metabolism. Besides being of exogenous origin, cholesterol is probably also synthesized to some extent by the body, since it has been found that the elimination can exceed the intake. Experiments on white rats by Randles and Knudson (72) give experimental evidence to show that the organism of the white rat is able to synthesize cholesterol. The diet was cholesterol free and the experiments were well controlled. The fate of cholesterol in metabolism is obscure. It seems to be closely related to cholic acid, which occurs in the two bile acids, glycocholic and taurocholic acids. The cholesterol contained in the bile after it reaches the intestine is mostly reabsorbed. A part is secreted unchanged in the bile. Coprosterol is formed by the reducing action of bacteria on cholesterol in the lower intestine. By processes of oxidation or reduction the body can form various products from cholesterol.

Cholesterol has been the subject of much investigation of late, particularly in regard to the antirachitic properties of irradiated cholesterol. Considerable productive work has been done on the physical and chemical properties of cholesterol. Nevertheless, there still remains a large field for future research work.

Cholesterol is a white crystalline substance insoluble in water, but soluble in the organic solvents alcohol, ether, chloroform, petroleum ether and benzene. It belongs to the class of sterols, which are aromatic alcohols of high mole-

cular weights, closely related to the terpenes. The formula, which remains uncertain, has within the last few years been considered to be $C_{27}H_{46}O$ or $C_{27}H_{45}OH$ (89). This is nearly the formula, $C_{26}H_{44}O$, given it by Berthelot in 1856. Windaus (89) gave its structural formula as:



The arrangement of the four rings is fairly well established, but the structure of the side chain is less well established.

Sources of commercial cholesterol are: gall stones, whale oil, cod liver oil, fish oil and pig brain. Gall stones are composed chiefly of cholesterol and may therefore be used as a source of it. The gall stones are pulverized, mixed with alcohol-ether, some potassium hydroxide added, and the mixture refluxed for two hours. On cooling, the cholesterol precipitates out. The bile pigment may be washed out with alcohol; the precipitate of cholesterol is dried and recrystallized from chloro-

form to constant melting point. In preparing cholesterol in the above manner from human gall stones, I was able to obtain a melting point of 147°C. (uncorrected); but even after repeated crystallizations the melting point remained unchanged. The melting point of commercial cholesterol is 148.5°C. This latter may be purified by a method proposed by Anderson (1). Material prepared by him using the special purification process has a melting point of 150- 151°C. (corrected). In his article, Anderson calls attention to the fact that cholesterol preparations obtained from different sources show slight differences in physical properties. Cholesterol, as found, is laevorotatory, a_D at 15°C., calculated from a solution containing 2 grams of cholesterol in 100 cc. ether, being -31.1 (Hesse). (52) Various workers have subjected cholesterol to irradiation and studied the effect on its spectrum. The author is now engaged in studies on the specific rotation and refractive index of cholesterols from various sources.

Cholesterol is a secondary alcohol, and as such it is not hydrolyzed by alkalies although its esters are, hence cholesterol itself is a constituent of the "Unsaponifiable fat fraction". It is an unsaturated compound since it contains one double bond. Therefore, it can absorb two atoms of iodine or bromine and from this a halogen number of cholesterol can be determined. The calculated theoretical iodine number of cholesterol is 64.7

Windaus (87) discovered that free cholesterol is precipitated by the alkaloid digitonin, while the esters are not, and investigation showed in alcoholic solution the

precipitation is almost quantitative. On this basis a quantitative method was developed (88). The procedure is as follows: the cholesterol containing substance is extracted with 50 cc. volume of 95 % alcohol, the free cholesterol is precipitated by the addition of a 1 % solution of digitonin in 90 % alcohol. It is allowed to stand for several hours, then is filtered through a weighed Gooch crucible, washed with cold alcohol and with ether, dried at 100° C., and weighed. The weight multiplied by the factor 0.2431 gives the weight of cholesterol in the amount of solution taken. To determine total cholesterol it is necessary to saponify with sodium hydroxide.

The cholesterol esters may be quantitatively determined as described in the method of Windaus (88). The filtrate from the above is concentrated, water and petroleum ether are added, and the mixture is shaken. This dissolves out the eholesterol esters, fats, lipids, and leaves the digitonin in solution. Separate, saponify the cholesterol esters, precipitate with digitonin, and then carry out the remainder of the procedure as before. A correction of 0.0016 gm. per 10 cc. of solution taken is added to the weight of digitonin cholesteride before it is multiplied by the factor 0.2431 The correction is for the amount of digitonin cholesteride left in solution, since the compound is not absolutely insoluble in alcohol. Mueller (69) in repeating Windaus' procedure concluded that the amount of digitonin cholesteride lost in washing the precipitate to be inconsequential.

Bloor published methods for the separate determination of cholesterol and cholesterol esters in small amounts of blood (13, 16) based upon the method of Windaus. He adapted it for use on the alcohol-ether extracts of blood. Free cholesterol is determined gravimetrically (13), by precipitating the cholesterol with digitonin. Ten cc. of the alcohol-ether extract are used for each determination. For the estimation of the cholesterol in combination as ester, it was found better to use 20 cc. of the extract. Cholesterol is precipitated with 1 cc. of a 1 % solution of digitonin in alcohol. It is then evaporated to dryness on the electric stove, and the dried residue extracted with petroleum ether, which dissolves out the cholesterol esters, but not the digitonin precipitate. The combined extracts are evaporated to dryness, the esters taken up with chloroform as in the method for total cholesterol. The chloroform extracts are made up to a volume of 5 cc. and the cholesterol determined colorimetrically.

Bloor's method published in 1922 receives the most general useage. An alcohol-ether extract of blood is made in the manner already described. Aliquot portions of the extract are taken for the determination. Ten cc. portions of the alcohol-ether extract are measured into small beakers of 100 cc. capacity. Saponification is effected by the addition of 0.1 cc. of concentrated sodium hydroxide, made from sodium. The mixture is then evaporated on the water bath until only a few drops remain. The alkali is then partially neutralized by the addition of 0.1 cc. of dilute sulphuric acid, and the liquid well mixed and distributed

over the bottom of the beaker. It is then evaporated just to dryness. After cooling, 10 cc. of redistilled chloroform are added to each beaker, allowing the contents to stand for ten minutes to insure complete extraction. The chloroform extract is then poured through a small filter into another small beaker, and the extraction repeated twice with 5 cc. portions of redistilled chloroform. The combined chloroform extracts are then evaporated down to a volume of 2 to 3 cc., poured into a ten cc. graduated glass-stoppered cylinder, and made up to 5 cc. with the chloroform washings from the beaker. The cholesterol may then be determined colorimetrically by use of the Lieberman-Burchard reaction, which is as follows:- to the contents of the graduated cylinder are added 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulphuric acid, the cylinder is then stoppered, the contents well mixed, and placed in the dark for 30 minutes. At the end of that time it is compared in the colorimeter with a standard, which was set up at the same time as the unknown. The standard solution of cholesterol contains 1 mgm. of cholesterol in 5 cc. of chloroform. The commercial preparations of cholesterol may be used for the standard solutions for colorimetric comparison. The standard solution is made from a stock solution containing 0.2000 gm. of cholesterol in 200 cc. chloroform, by diluting 20 cc. of the stock solution to a volume of 100 cc. with chloroform. Therefore, 1 cc. contains 0.2 mgm. cholesterol.

There are of course other methods for the estimation of cholesterol in blood. A procedure (70) used by some investigators is that of Myers and Wardell. This method is based upon drying the blood by mixing it with plaster of paris, refluxing with chloroform to extract the cholesterol, and subsequently using colorimetric methods for the determination of the amount of cholesterol in the sample of blood taken. However, this procedure has not the advantages of Bloor's method as it is more time consuming, and requires a special preparation of material. Bloor's procedure is advantageous in that it may be applied to the same alcohol-ether extract of blood as is used for the other lipid determinations.

A slight modification of Bloor's method, developed by McClure and Huntsinger (64), was necessitated by the fact that it was frequently difficult to saponify the cholesterol esters in the presence of solvents other than alcohol. For this reason, the original alcohol-ether extract described by Bloor was evaporated to a bulk of a few cc. in order to drive off the ether. To this was added 15 cc. of redistilled alcohol, and 0.1 cc. of the sodium hydroxide solution, and the mixture boiled for ten minutes on the electric hot plate. After this preliminary saponification, the remainder of the procedure was carried out as described by Bloor.

Sackett (74) published a modification of Bloor's method for the determination of cholesterol in whole blood or blood serum. The advantages claimed for it as compared to Bloor's method are that it requires less alcohol and ether, less blood, and less time in carrying out the

test. The procedure is as follows: 9 cc. of alcohol and 3 cc. of ether are pipetted into a 15 cc. graduated centrifuge tube, and mixed by inverting. Then 0.2 cc. of whole blood or plasma are run in, the tube is then corked tightly and shaken vigorously for one minute. The stoppered tube is placed on its side for 30 minutes, and is then centrifuged rapidly for 3 minutes, after which the supernatant liquid is decanted into a small beaker, and evaporated just to dryness on the water bath. The cholesterol is extracted twice for about two minutes with 2 cc. of chloroform, and decanted into a 10 cc. glass stoppered graduated cylinder, and made up to a volume of 5 cc. The procedure from this point on is the same as previously described. The cholesterol is quantitated colorimetrically using the Lieberman-Burchard reaction. This method as checked against Bloor's procedure gives very good results. For example, Sackett reports that a specimen giving a cholesterol value of 211.7 mgm. using Bloor's method gave a value of 210.1 mgm. using his modification.

A simplified method for the determination of cholesterol in blood was published in 1924 by Leiboff (53). The values obtained are generally slightly higher than those obtained by other methods. The procedure of Leiboff has the advantage of using only a small amount of blood (0.25 cc.) and since there is no transfer of material there is no possible loss. Five cc. of chloroform are put into the extraction tube and the filter paper disc is drop-

ped into the tube. The constricted portion of the tube holds the disc in place, and also permits accurate dilution. The tube resembles somewhat a blood sugar tube. 0.25 cc. of blood is pipetted on the filter paper disc, which immediately absorbs the blood. The extraction tube is then attached to a reflux condenser and immersed in a beaker of hot water placed over a small electric stove. The water in the beaker should be above the level of the chloroform in the tube. The extraction is continued for thirty minutes, after which the tube is detached from the condenser and the disc removed. When cool, chloroform is added to the 5 cc. mark. The colorimetric estimation is then carried out as previously described.

In a comparison of colorimetric and gravimetric methods, Mueller (69) states that either is accurate, although the gravimetric methods are superior. The standard gravimetric procedure is the digitonin precipitation method already described. The colorimetric procedures give results too high for true cholesterol, because they include other ether and chloroform soluble substances, whereas digitonin determinations are more nearly correct. When time and material are available gravimetric methods usually give the best results, but in work where both factors are limited the colorimetric methods are often preferable.

Of all the methods proposed for the estimation of cholesterol in blood, that of Bloor is that of most general use. The efficiency of the procedure proposed by Bloor for the separation and estimation of cholesterol

and fatty acids of known concentration. Preliminary experiments showed that as much as 25 % of the fatty acids might fail to be recovered unless alcohol, freshly distilled over sodium hydroxide, was used. It was also found necessary to remove alcohol from the chloroform, otherwise some of the soaps were lost during the process of separating the cholesterol from the fatty acids. Such findings as these showed that only highly purified reagents should be used. It was also found that ether interfered with the saponification, as has already been stated above. For this reason the ether was removed by evaporation before attempting saponification. The method showed, however, that the method is highly satisfactory when carried out under proper conditions.

The normal values for blood cholesterol are given by Bloor as 210 to 240 mgm. per 100 cc. blood. Denis (32) reports normal values as between 190 to 250 mgm. McClure and Huntsinger (64) found that the normal values range between 120 to 200 mgm., but are usually between 140 to 180 mgm. In pathological cases, the cholesterol value may or may not vary from the normal. Increases in cholesterol content have been reported in hyperlipemia, diabetes (24), obstructive jaundice, and pregnancy (32). Low values have been reported in anemia (19, 39).

The Iodine Numbers of Fatty Acids.

The iodine number of a fat or fatty acid can be determined according to standard methods using one of the standard iodine solutions, such as those proposed by Hanus, Hübl or Wijs. A weighed sample of the fat is dissolved in chloroform, a definite volume of iodine solution is added, the contents of the flask are mixed, the flask is tightly stoppered and placed in the dark for a given time. At the end of this period, a measured amount of potassium iodide solution is added, water is added, and it is titrated against tenth normal sodium thiosulphate, which has been standardized against dichromate solution. Controls are run at the same time. The calculation depends upon the titration difference between the controls and the test, a value for the thiosulphate solution in terms of iodine, and the weight of the sample taken.

A micromethod for the determination of the iodine numbers of the fatty acids was devised by McClure and Huntsinger (64). The following procedure is used. Blood is extracted with alcohol-ether as previously described, (Bloor's method of extraction). 80 cc. of the filtered extract are pipetted into a beaker of 250 cc. capacity, and evaporated on the steam bath to a volume of a few cc. Then 15 cc. of redistilled alcohol and 0.8 cc. of the pure sodium hydroxide are added, and the whole boiled on the electric hot plate for ten to fifteen minutes. Following this period of saponification it is evaporated just to dryness on the steam bath. After cooling, the dry residue

is extracted three times in the cold with 20 cc. of redistilled chloroform. Each portion of the chloroform is poured through a 7 cm. filter paper into a 100 cc. erlenmeyer flask. This extraction with chloroform completely frees the residue from the cholesterol. The chloroform extracts of cholesterol obtained are used in the determination of the iodine numbers of cholesterol, to be described later. The residue contains the fatty acids in the form of soaps. These are converted into fatty acids by the addition of 10 cc. of redistilled alcohol containing 15 % of concentrated hydrochloric acid. The whole is then boiled for ten to fifteen minutes on the electric hot plate, after which the alcohol and acid are driven off by evaporation to dryness on the steam bath. The fatty acids are then extracted from the residue in the beaker by three extractions each with 10 cc. portions of cold chloroform, allowing ten minutes for each extraction. The chloroform portions are decanted through the same filter paper as that used in separating out the cholesterol, and are collected in an erlenmeyer flask of 100 cc. capacity. The chloroform solution of fatty acids thus obtained is evaporated just to dryness on the steam bath.

The fatty acids are redissolved in 1 cc. of chloroform in the flask and 2 cc. of Hanus' iodine solution are added. The flask is stoppered, its contents mixed by rotation, and is then placed in the dark for one hour. The unabsorbed iodine is determined by adding 0.5 cc. of a 15 % solution of potassium iodide, 10 cc. of distilled water, and titrating with 0.01 N sodium thiosulphate solution using starch

solution as an indicator. Three control flasks containing 1 cc. of chloroform and 2 cc. of Hanus' iodine solution are run with each determination. At the same time the values of the solutions used are checked, using the usual cross titrations and a standard solution of potassium dichromate. From the weight of the iodine bound by the fatty acids in this procedure and the weight of the fatty acids as found by Bloor's nephelometric method, the iodine number is calculated.

The Iodine Number of Cholesterol.

Since there is one double bond in the cholesterol molecule it is capable of absorbing two atoms of halogen. Work on the iodine number of the cholesterol in blood has been carried out by McClure and Huntsinger (64) in studies on fat metabolism. These authors published a method for the estimation of the iodine numbers of both cholesterol and fatty acids in small amounts of blood. The cholesterol is extracted with chloroform as described above. The chloroform is evaporated off just to dryness on the steam bath. The cholesterol is then redissolved in 1 cc. of chloroform in the flask, and 2 cc. of Hanus' iodine solution are added. The flask is stoppered, its contents mixed by rotation, and it is then placed in the dark for one hour. The unabsorbed iodine is determined by adding 0.5 cc. of a 15 % solution of potassium iodide, 10 cc. of distilled water, and titrating with 0.01 N sodium thiosulphate solution, using starch solution as an indicator. Here, as in the determination of the iodine numbers of the

fatty acids, controls are run at the same time as the tests. The strengths of the iodine and the thiosulphate solutions are determined by the usual cross titrations and the use of a standard chromate solution. From the data obtained through this determination and the weight of the cholesterol in the blood as found by Bloor's method, the iodine number of cholesterol can be calculated.

Discussion.

Perusal of the literature, which was undertaken for the purposes of the present thesis, shows a vast accumulation of valuable data relating to the biochemistry of lipids, including the fats. Nevertheless, as a glance at the classifications given in this paper show, not enough is known to permit more than an arbitrary classification of the lipids present in the animal organism. Indeed, only since 1908 have satisfactory methods for their analysis been developed. Far more must be known of the physical and chemical properties of the lipids before an adequate classification can be developed. Therefore, the one offered by the author is for descriptive purposes only.

Methods of lipid analysis are (1) macro methods, and (2) micro methods. Macromethods include gravimetric and volumetric procedures. Micromethods are largely colorimetric, but may be nephelometric or volumetric. The macromethods frequently have the advantage of greater accuracy. On the other hand, they require relatively large amounts of material and for this reason frequently are not

applicable to the study of phenomena occurring in the living organism. The micro methods have the advantages of rapidity and the use of very small amounts of material. For this reason they permit numerous observations to be made upon the animal without causing physical disturbances. However, they are usually only relatively accurate. Nevertheless, by their use a great deal of valuable data concerning both normal and abnormal lipid metabolism has been collected.

There is no method for the quantitation of fatty acids, which is free from criticism. The gravimetric procedures call for drying, the accomplishment of which is somewhat uncertain. The volumetric methods calculate the weight of fatty acids from the degree of acidity. Obviously, such a procedure gives results of relative accuracy only. The very useful nephelometric micromethod devised by Bloor is inelastic, because the same degree of hydrogen ion concentration is used, and inaccurate because of the limitations of the method. The degree of turbidity which is developed will vary according to the fatty acids which happen to be present. The method will give relatively comparable results when the fatty acids are mixtures of oleic and palmitic. However, in the presence of other types of fatty acids the results may be widely erroneous. Bloor's latest oxidation method is theoretically much more accurate and undoubtedly much more elastic. It has the disadvantage of being technically more difficult. Which method is preferable depends upon the character of the results demanded by the particular investigation. Where time and material permit,

the macro methods proposed by Kumagawa and Suto are preferable. Many types of biological studies can only be accomplished by use of micromethods, of which those developed by Bloor are undoubtedly the most satisfactory.

Phosphatides are highly important substances in metabolism, nevertheless, the functions they preform are only imperfectly known. The accuracy with which phosphatides may be quantitated depends upon the method for determining their phosphorus content. The latter can be ascertained with a high degree of accuracy by gravimetric means. On the other hand, comparison of reported figures obtained by the various micromethods and the author's personal experiences with them indicate that the analytical results are not uniform. Such observations have led the author to conclude that the method of Fiske is the best available at this time.

For usual biochemical studies the colorimetric method developed by Bloor is superior for the estimation of cholesterol. Where time and material permit, the gravimetric method of Windaus gives somewhat more accurate results. By either method it must be considered that cholesterol is apt to be contaminated by the presence of other sterols. Just in how far any of the methods will permit differentiation of these substances is unknown. It is quite possible that in the estimation of cholesterol, as ordinarily extracted from biological material, other sterols are concomitantly determined.

Summary.

1. For fatty acids Bloor's methods have been found to be most satisfactory. The oxidation method is the more accurate and more time consuming. For rapid clinical analyses the nephelometric method is useful.

2. For lecithin the procedure of Fiske and Subbarow is superior.

3. For cholesterol the colorimetric method of Bloor is dependable.

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